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(54) Title: **ANTI-SENSE POLYNUCLEOTIDE THERAPY FOR GASTRIN-PROMOTED TUMORS**

(57) **Abstract:** A therapeutic method for the treatment of patients suffering from a gastrin-promoted tumor; a tumor comprising cells bearing a gastrin receptor; or a tumor of the gastrointestinal (GI) tract, a pancreatic tumor, a medullary thyroid carcinoma (MTC), a tumor of the lung, a glioblastoma, an ovarian tumor, or a tumor of neuroendocrine origin is provided. The method includes administering to a patient a DNA molecule comprising a gastrin anti-sense sequence operatively linked to a regulatory sequence that expresses gastrin anti-sense RNA to the patient, and also administering a chemotherapeutic agent. The method is especially useful for the treatment of patients with advanced stage disease, and for patients having undergone prior surgery, radiotherapy or chemotherapy treatment.

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## ANTI-SENSE POLYNUCLEOTIDE THERAPY FOR GASTRIN-PROMOTED TUMORS

BACKGROUND

Pancreatic adenocarcinoma remains a formidable disease responsible for six percent of all cancer deaths and associated with limited treatment options (Gilliam AD, & Watson SA. Emerging biological therapies for pancreatic carcinoma. *Eur J Surg Oncol.* 28(4): 370-8, 2002). Surgical resection rates are low due to late presentation and diagnosis of the disease. The first line treatment for patients with locally advanced or metastatic disease is gemcitabine, a nucleoside analogue, which has been associated with a clinical response rate of 23.8% (Carmichael J. Clinical response benefit in patients with advanced pancreatic cancer. Role of gemcitabine. *Digestion* 58: 503-507, 1997; Burris HA, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol.* 15(6): 2403-13, 1997).

Gastrin increases cell migration and activates focal adhesion kinase (Daulhac L, et al. Gastrin stimulates the formation of a p60Src/p125FAK complex upstream of the phosphatidylinositol 3-kinase signaling pathway. *FEBS Lett* 445(2-3): 251-5, 1999; Pagliocca A, et al. Stimulation of the gastrin-cholecystikinin(B) receptor promotes branching morphogenesis in gastric AGS cells. *Am J Physiol Gastrointest Liver Physiol* 283(2): G292-9, 2002).

Serum gastrin levels have been suggested as a marker of activation of the gastrin gene in tumor tissue (Siddheshwar RK, et al. Plasma levels of progastrin but not amidated gastrin or glycine extended gastrin are elevated in patients with colorectal carcinoma. *Gut* 2001;48(1):47-52; Muhammad KB, et al. Expression of tissue progastrin, CCK-B/gastrin receptor and cell proliferation in hepatic metastases from colorectal cancer. *Gastroenterology* 2003, 124(S1):M1079). Serum gastrin levels, particularly progastrin levels, were detectable and significantly higher than those in patients with resectable disease.

Gastrin can increase the level of anti-apoptotic proteins such as Bcl-2 (Konturek PC, et al. Influence of gastrin on the expression of cyclooxygenase-2, hepatocyte growth factor and apoptosis-related proteins in gastric epithelial cells. *J Physiol Pharmacol* 54(1): 17-32, 2003; Konturek PC, et al. Expression of hepatocyte growth factor, transforming growth factor alpha, apoptosis related proteins Bax and Bcl-2, and gastrin in human gastric cancer. *Aliment Pharmacol Ther* 15(7): 989-99, 2001). Gastrin has also been shown to enhance phosphorylation of PKB/Akt in response to serum withdrawal in the rat pancreatic adenocarcinoma cell line, AR42J (Todisco A et al. 2001 *supra*). The phosphorylated form of PKB/Akt inactivates a range of pro-apoptotic factors

including caspase-9, *Bad* and *fork-head/winged-helix*, transcription factors important in the transcription of the cell death ligand, *fas*, and activates the anti-apoptotic IKK/NF $\kappa$ B cascade (Cross TG, et al. Serine/threonine protein kinases and apoptosis. *Exp Cell Res* 256(1): 34-41, 2000).

Treatment with a neutralizing antiserum directed against both amidated and glycine-extended gastrin was shown to significantly reduce the *in vitro* basal growth of both PAN1 and BXPC3 cell lines and to induce synergistic inhibitory effects with the chemotherapeutic agents, gemcitabine and taxotere.

The gastrin gene is expressed widely in pancreatic adenocarcinomas. The role of gastrin and the gastrin/CCK-2 receptor (also known as CCK-B) in human pancreatic carcinogenesis has remained a focus of investigation since the first report identifying gastrin as a growth factor of human pancreatic cancer by Smith *et al* (Smith JP, et al. Identification of gastrin as a growth peptide in human pancreatic cancer. *Am J Physiol* 268(1Pt2): R135-41, 1995). Gastrin expression has been implicated in pancreatic weight gain through exocrine stimulation of the CCK-2 receptor, as demonstrated by increased weight of the pancreas in a mouse model expressing CCK-2 receptor (Yen TW, et al. The gastrin receptor promotes pancreatic growth in transgenic mice. *Pancreas* 24(2): 121-9, 2002). Also, when a mouse transgenic for the CCK-2 receptor was crossed with a transgenic hypergastrinaemic mouse malignant transformation resulted in three of twenty offspring (Clerc P, et al. Expression of CCK2 receptors in the murine pancreas: proliferation, transdifferentiation of acinar cells, and neoplasia. *Gastroenterology* 122(2): 428-37, 2002).

The CCK-2 receptor and gastrin are both co-expressed in human pancreatic adenocarcinoma specimens (Goetze JP, et al. Closing the gastrin loop in pancreatic carcinoma: coexpression of gastrin and its receptor in solid human pancreatic adenocarcinoma. *Cancer* 88(11): 2487-94, 2000; Caplin M, et al. Expression and processing of gastrin in pancreatic adenocarcinoma. *Br J Surg.* 87(8): 1035-40, 2000) and cell lines (de Weerth A, et al. Human pancreatic cancer cell lines express the CCKB receptor. *Hepatogastroenterology* 46(25): 472-8, 1999; Monstein HJ, et al. Differential expression of gastrin, cholecystokinin-A and cholecystokinin-B receptor mRNA in human pancreatic cancer cell lines. *Scand J Gastroenterol* 36(7): 738-43, 2001; Mandair KK, et al. Cholecystokinin receptors in human pancreatic cancer cell lines. *Eur J Cancer* 34(9): 1455-9, 1998; Smith JP, et al. Quantitative analysis of gastrin mRNA and peptide in normal and cancerous human pancreas. *Int J Mol Med* 2(3): 309-315, 1998) at both the gene and protein levels. Secretion of gastrin protein was identified in BXPC3 and seven additional pancreatic cell lines together with immuno-cytochemical confirmation of gastrin

expression in human pancreatic cancer specimens but not associated normal tissue (Smith JP, et al. Gastrin regulates growth of human pancreatic cancer in a tonic and autocrine fashion. *Am J Physiol* 270(5 Pt 2): R1078-84, 1996).

Using a specific radioimmunoassay, Goetz *et al* confirmed the expression of amidated gastrin in fourteen of nineteen carcinomas tested (Goetze JP et al. *supra*). A second study detected mainly precursor gastrin forms; progastrin and glycine-extended gastrin (Caplin M et al. *supra*). Glycine-extended gastrin peptides were also shown to be secreted by the rat pancreatic cell line, AR42J (Negre F, et al. Autocrine stimulation of AR4-2J rat pancreatic adenocarcinoma cell growth by glycine-extended gastrin. *Int J Cancer* 66(5): 653-8, 1996).

The CCK-2 receptor increases expression of key signalling pathways following activation by externally applied gastrin. In the rat pancreatic cell line, AR42J, the pathways activated include those involving mitogen activated protein kinase (MAPK) (Dabrowski A, et al. Stimulation of both CCK-A and CCK-B receptors activates MAP kinases in AR42J and receptor-transfected CHO cells. *Digestion* 58(4): 361-7, 1997) and also protein kinase B/Akt (PKB/Akt). The latter protein kinase imparts resistance to apoptotic stimuli (Todisco A, et al. Molecular mechanisms for the antiapoptotic action of gastrin. *Am J Physiol Gastrointest Liver Physiol*. 280(2): G298-307, 2001).

Gastrin has now been confirmed as a central growth factor for malignancies of the gastro-intestinal (GI) tract having proliferative and anti-apoptotic effects possibly indirectly through increasing transcription of ligands of the EGF receptor (Miyazaki Y, et al. Gastrin induces heparin-binding epidermal growth factor-like growth factor in rat gastric epithelial cells transfected with gastrin receptor. *Gastroenterology* 116(1): 78-89, 1999; Varro A, et al. Gastrin-cholecystokinin(B) receptor expression in AGS cells is associated with direct inhibition and indirect stimulation of cell proliferation via paracrine activation of the epidermal growth factor receptor. *Gut* 50(6): 827-33, 2002), the REG protein (Fukui H, et al. Regenerating gene protein may mediate gastric mucosal proliferation induced by hypergastrinemia in rats. *Gastroenterology* 1998;115(6):1483-93) and COX-2 (Guo YS, et al. Gastrin stimulates cyclooxygenase-2 expression in intestinal epithelial cells through multiple signaling pathways. Evidence for involvement of ERK5 kinase and transactivation of the epidermal growth factor receptor. *J Biol Chem* 2002;277(50):48755-63).

Treatments directed to interaction with serum gastrin, including CCK-2 receptor antagonists and the gastrin vaccine, G17DT, and have been proven to be effective clinically (Heneghan MA, et al. Use of a novel CCKB/gastrin receptor antagonist, Gastrazole (JB5008) in patients with advanced pancreatic

adenocarcinoma. Results of an open labelled pilot trial. *Gastroenterology* 2001;120(S1):3108; Brett BT, et al. Phase II study of anti-gastrin-17 antibodies, raised to G17DT, in advanced pancreatic cancer. *J Clin Oncol* 2002;20(20):4225-31; Smith AM, et al. Phase I/II study of G17-DT, an anti-gastrin immunogen, in advanced colorectal cancer. *Clin Cancer Res* 2000;6(12):4719-24).

In previous studies, Smith et al showed that BXPC3 cells transfected with gastrin antisense oligonucleotides exhibited a greater than 30% reduction in final tumor weight when transplanted orthotopically (Smith JP, et al. Antisense oligonucleotides to gastrin inhibit growth of human pancreatic cancer. *Cancer Lett* 135(1): 107-12, 1999). However, in this latter study there was no suggestion of treatment with a combination of gastrin antisense oligonucleotides and cytotoxic agents.

The available therapeutic options for pancreatic cancer are limited at present and new approaches are needed to either work in concert with existing therapeutic modalities and/or to improve treatment for patients with advanced disease for which there are currently no viable options.

#### SUMMARY OF THE INVENTION

Gastrin is known to be a mediator of neoplastic transformation and, in many cases, of tumor growth and proliferation. The present invention provides a variety of methods employing anti-sense intervention in conjunction with chemotherapy to prevent tumor growth, proliferation and maintenance and to sensitize the tumor to chemotherapeutic agents.

The present invention provides a much needed novel and highly effective method of treating a patient suffering from: (1) a tumor of the of the gastrointestinal (GI) tract, a pancreatic tumor, a medullary thyroid carcinoma (MTC), a tumor of the lung, a glioblastoma, an ovarian tumor, or a tumor of neuroendocrine origin; (2) a gastrin-promoted tumor; or (3) a tumor comprising cells bearing a gastrin receptor. The method includes administering to the patient a therapeutically effective amount of a nucleic acid molecule comprising a gastrin anti-sense sequence operatively linked to a regulatory sequence that expresses gastrin anti-sense RNA, the treatment also includes administering a chemotherapeutic agent to the patient.

The invention also provides a method of treating a patient suffering from any of the above listed tumors, the method includes administering to the patient a therapeutically effective amount of a single stranded nucleic acid molecule (either RNA or DNA), comprising a gastrin anti-sense sequence, the treatment also includes administering a chemotherapeutic agent to the patient.

The present invention yet further provides a gastrin anti-sense expression vector, that includes the human gastrin gene sequence from about nucleotide 3 to about nucleotide 383 of SEQ ID NO:5, and/or the complement of the human gastrin gene sequence from about nucleotide 3 to about nucleotide 383 of SEQ ID NO:5, wherein the vector expresses the anti-sense sequence complementary to the human gastrin gene sequence from about nucleotide 3 to about nucleotide 383 of SEQ ID NO:5.

The invention also provides a method of making a gastrin anti-sense expression vector. The method includes incorporating the human gastrin gene sequence from about nucleotide 3 to about nucleotide 383 of SEQ ID NO:5, and/or the complement of the human gastrin gene sequence from about nucleotide 3 to about nucleotide 383 of SEQ ID NO:5 into an expression vector, wherein the vector expresses the anti-sense sequence complementary to the human gastrin gene sequence from about nucleotide 3 to about nucleotide 383 of SEQ ID NO:5.

The invention further provides a pharmaceutical composition comprising a nucleic acid molecule that includes a gastrin anti-sense sequence operatively linked to a regulatory sequence that expresses a gastrin anti-sense RNA, and a pharmaceutically acceptable carrier.

The invention yet further provides a pharmaceutical composition that includes a nucleic acid molecule comprising a gastrin anti-sense sequence, and a pharmaceutically acceptable carrier.

#### BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Gastrin expression of the two test pancreatic adenocarcinoma cell lines in comparison to a panel of human pancreatic adenocarcinoma specimens from patients with resectable disease. A) Gene expression was determined by real time PCR and each observation is the mean of 2 cDNA samples assessed in duplicate (standard deviations do not show with current scale of graph). B) Progastrin immuno-reactivity as measured by image analysis. Each observation is the mean percent staining (with standard deviations shown) following assessment of ten separate fields per section. Percent staining was calculated following assessment of ten separate fields per section by image analysis.

Figure 2: Gastrin expression of PAN1 vector control and PAN1 gastrin antisense cell lines. A) Gastrin gene expression determined by real time PCR. \*  $p < 0.001$ , Mann Whitney U,  $n=3$  replicates of two separate cDNA samples. B) Immunocytochemical staining using progastrin antiserum raised against the amino terminus. C) Clonogenicity Assay with PAN1 VC and PAN1 AS cell lines in: (i) 1% serum-containing medium, (ii) 0.2% serum-containing medium. Ten replicates per time

point and results are representative of a single assay that was repeated three times. \*\* $p < 0.01$ , Student's t-test.

Figure 3: Typical dose response curve for gemcitabine and taxotere in the PAN1 vector control and PAN1 gastrin antisense cell lines as determined by MTT uptake. A) Gemcitabine B) Taxotere. \* $p < 0.0001$  as assessed by one-way analysis of variance comparing MTT uptake in PAN1 VC versus PAN1 AS cells.

Figure 4: Effect of taxotere and gemcitabine on the orthotopic growth of PAN1 VC and PAN1 AS cells in the pancreas. A) Final tumor cross-sectional area ( $\text{mm}^2$ ). \* $p = 0.04$  \*\* $p = 0.000$  when compared to PAN1 VC. \* $p = 0.013$  and \*\* $p = 0.009$  when compared to PAN1 AS as assessed by ANOVA. B) Final tumor weight. \* $p = 0.000$  \*\* $p = 0.115$  \*\*\* $p = 0.000$  when compared to PAN1 VC. \* $p = 0.03$  and \*\* $p = 0.006$  when compared to PAN1 AS as assessed by ANOVA.

Figure 5: A comparison of caspase-3 levels in PAN1 VC and PAN1 AS cell lines following treatment with gemcitabine or taxotere. Cells were incubated for 4 hours with  $20\mu\text{g/ml}$  taxotere or  $250\mu\text{g/ml}$  gemcitabine. Cumulative results of two separate assays performed in duplicate. \*NS, \*\* $p = 0.032$ , \*\*\* $p = 0.013$  compared to PAN1 VC cells treated with the same conditions, one-way analysis of variance.

Figure 6: PKB/Akt expression of PAN1 vector control and PAN1 gastrin antisense cell lines following treatment with either gastrin, taxotere or gemcitabine. A) PKB/Akt phosphorylation in PAN1 VC, PAN1 AS and AR42J (positive control) cells in the absence and presence of gastrin-17 ( $10\text{nM}$  assessed up to 60mins incubation). B) PKB/Akt phosphorylation in PAN1 VC and PAN1 AS following treatment with the cytotoxic agents, gemcitabine ( $250\mu\text{g/ml}$ ) and taxotere ( $20\mu\text{g/ml}$ ) after 1 hour treatment. Representative blots were repeated twice.

#### DETAILED DESCRIPTION OF THE INVENTION

Abbreviations used herein: Mouse anti-gastrin mAb - mouse monoclonal anti-gastrin antibody; DAB - diaminobenzidine; EDTA - ethylenediaminetetraacetic acid; FBS - fetal bovine serum; GAPDH - glyceraldehyde phosphate dehydrogenase; G17 - Gastrin-17;  $\text{IC}_{50}$  - concentration inducing 50% inhibition; MAPK - mitogen activated protein kinase; MTT - ethyl thiazoyl tetrazolium; PAN1 AS - PAN1 clone transfected with gastrin anti-sense plasmid; PAN1 VC - PAN1 cells transfected with control plasmid; PBS - phosphate buffered saline; PCR - polymerase chain reaction; PKB/Akt - protein kinase B/Akt.

DEFINITIONS: As used herein the term 'patient' refers to a human or other mammal in need of cancer treatment or prophylaxis.

Gastrin hormone exists in several molecular forms. Gastrin 17 (G17) is a seventeen amino acid peptide having a pyroglutamic acid residue at the N-terminus

and an amidated phenylalanine residue at the C-terminus. Gastrin 34 (G34) is a thirty-four residue peptide comprising the amino acids of gastrin 17 at the C-terminus, with an additional seventeen amino acid residues at the N-terminus. Glycine-extended gastrin 17 (G17-Gly) has the amino acid sequence of G17 with an additional glycine residue at the C-terminus. Similarly, glycine-extended gastrin 34 (G34-Gly) has the amino acid sequence of G34 with an additional glycine residue at the C-terminus.

A 'gastrin-promoted tumor' as used herein means a tumor that is stimulated to proliferate by one or more of the gastrin hormone forms, such as for instance, gastrin 17, gastrin 34, glycine-extended gastrin 17, and/or glycine-extended gastrin 34.

The gastrin-promoted tumor may be stimulated by a gastrin hormone form produced by another tissue (paracrine stimulation), or by cells of the tumor tissue itself (autocrine stimulation). Gastrin 17, gastrin 34 and the glycine-extended gastrin hormone forms each bind different receptors which are found on normal gastrin-stimulated cells and/or on gastrin-stimulated tumor cells.

Tumors treatable by the methods of the present invention include tumors of the gastrointestinal (GI) tract. The present methods are particularly suitable for treatment of tumors of the upper GI tract, such as for instance, tumors of the esophagus (including Barrett's esophagus), or stomach, particularly tumors of gastric tissue. The tumors treatable by the methods of the present invention include tumors that are classified as including metaplasias (i.e. abnormal replacement of cells of one type by cells of another) and dysplasias (i.e. abnormal growth or development of organs or cells).

The methods of the present invention are also suitable for treating tumors of the of the intestinal tract, such as for instance, tumors of the small intestine, colonic adenomas and tumors of the colorectal tract. Moreover, the methods of the present invention are especially useful in the treatment of pancreatic tumors.

The methods of the present invention are also applicable in the treatment of other tumors, including gastrin-promoted tumors susceptible to gastrin anti-sense treatment; such tumors include, but are not limited to medullary thyroid carcinomas (MTC), tumors of the lung, such as small cell lung cancer (SCLC), ovarian tumors, glioblastomas and tumors of neuroendocrine origin.

The methods of the present invention are especially useful in the treatment of advanced stage tumors, and tumors that are refractory to standard therapeutic approaches, including, surgery, radiation therapy and chemotherapy.

As used herein, an "advanced stage tumor" is a tumor at a stage of metastasis. The tumor may be characterized as at or beyond stage C of the



Whitmore-Jowett tumor staging system. Alternatively, an "advanced stage tumor" is characterized as a tumor at or beyond T3 of the TNM (Tumor, Node, Metastasis) staging system.

The Whitmore-Jowett staging system uses letters A-D to identify tumor stages. Stage A is where the tumor is well differentiated and confined to one site, or is moderately/poorly differentiated and present in more than one site. Stage B tumors are large enough to be palpably detected. Stage C tumors may involve almost an entire gland and may have spread a small distance beyond the original tissue. Stage D indicates widespread (metastatic) cancer.

The TNM staging system relies on characteristics of the tumor, regional lymph nodes, and metastases (where present). Stages T1-T4 relate to primary tumor characteristics: T1- tumor may not be palpable or visible by imaging, or may be detectable by needle biopsy; T2- tumor confined to a single tissue, up to whole tissue involvement; T3- tumor extends to surrounding tissue; T4- Tumor extensively present in other tissues. The regional lymph nodes are characterized as N0-N1, where N0 shows no regional lymph node involvement and N1 shows metastasis in regional lymph nodes. Similarly, the metastasis staging is from M0-M1, where M0 is no detectable distant metastasis and M1 corresponds to distant metastasis.

The methods of the present invention are particularly useful in the treatment of gastrin-promoted and gastrin-secreting tumors. Gastrin-secreting tumors are tumors that secrete gastrin (eg. gastrinomas), but may or may not be gastrin-promoted. Such tumors may provide gastrin as a paracrine growth factor for gastrin-promoted tumors at distant sites in the patient. In addition, the methods of the present invention are useful in the treatment of tumors that include cells bearing gastrin receptors. Tumor cells bearing gastrin receptors are susceptible to activation of proliferative pathways by gastrin. Anti-sense treatment causes reduction or complete ablation of gastrin expression and hence reduces or prevents this gastrin-promoted proliferation.

In one embodiment the present invention provides a method of treating a tumor of the gastrointestinal tract, a pancreatic tumor, a medullary thyroid carcinoma (MTC), a tumor of the lung, a glioblastoma, an ovarian tumor, or a tumor of neuroendocrine origin; the method includes administering to a patient in need thereof: a DNA molecule comprising a gastrin anti-sense sequence operatively linked to a regulatory sequence such that the DNA molecule expresses a gastrin anti-sense RNA in the tumor tissue, and further administering a chemotherapeutic agent to the patient.

In a preferred embodiment the present invention provides a method of treating a gastrin-promoted tumor, by administering to a patient in need thereof, a

DNA molecule comprising a gastrin anti-sense sequence operatively linked to a regulatory sequence such that the DNA molecule expresses a gastrin anti-sense RNA in the tumor tissue, and also administering a chemotherapeutic agent to the patient.

The DNA molecule expressing the gastrin anti-sense RNA may include any of the many publicly available regulatory sequences well known in the art that express gastrin anti-sense RNA when linked to the gastrin anti-sense sequence. Such regulatory sequences include, but are not limited to any of the many well known regulatory sequences for example: viral regulatory sequences (e.g. the constitutively expressing sequence from the cytomegalovirus, CMV immediate-early enhancer/promoter region; adenovirus promoters; Moloney-Murine Leukemia Virus, M-MuLV promoters; or any of the many other viral vectors well known in the art), and any of the readily available regulatory sequences that are constitutive or inducible in mammalian systems, such as for example, beta-actin or gamma-actin; the SV40 enhancer/promoter region sequence, and the herpes simplex virus thymidine kinase promoter: i.e. HSV TK promoter.

Two or more anti-sense nucleic acid molecules (whether RNA or DNA or a combination of one or more RNA molecules and one or more DNA molecules) may be administered to a patient suffering from a gastrin-stimulated tumor along with a chemotherapeutic agent according to the methods of the present invention in order to treat the tumor.

As used herein, a gastrin anti-sense nucleic acid molecule is an RNA or DNA molecule having a sequence complementary to at least a portion of the coding strand of the gastrin messenger RNA (mRNA) that is transcribed and processed from the gastrin gene. The anti-sense nucleic acid molecule also may comprise modified nucleotides that may increase the stability of the polynucleotide chain and/or provide resistance to nuclease digestion. Such modifications of the nucleotide base include, but are not limited to 5' and 3' blocking groups commonly used in polynucleotide synthesis in vitro, as well as substitution of one or more nucleotide bases, with a base such as for instance, inosine having an altered base pairing activity.

Examples of modifications of the phosphodiester backbone include for instance, a thioester linkage in place of one or more phosphodiester linkages of the nucleotide chain. The skilled artisan will immediately recognize the many well known modified nucleotides and linkages that may be usefully applied in the present invention.

The anti-sense nucleic acid molecule of the present invention need not be perfectly complementary to the sense strand of the gastrin RNA. Small numbers of

mismatches, deletions or additions are tolerated, provided that the anti-sense nucleic acid molecule expressed is effective in down-regulating gastrin gene expression. The number of mismatches may be, for instance, one, two, three or four mismatches for any given stretch of ten nucleotides in the anti-sense RNA molecule. The level of mismatch tolerated may be readily determined by one of ordinary skill in the art by the methods taught herein without undue experimentation.

The determination of the therapeutically effective amount of DNA molecule expressing the anti-sense RNA required for the practice of the present invention is readily determinable by one of ordinary skill in this art according to the methods herein disclosed. As used in the present specification, a therapeutically effective amount is that amount which when administered along with a chemotherapeutic agent according to the methods of the present invention results in stabilization of the tumor size and/or weight.

Preferably, the therapeutically effective amount is that amount which when administered along with a chemotherapeutic agent according to the methods of the present invention results in a reduction in size and/or weight of the tumor. The reduction in tumor size and/or weight is preferably at least about 10%; more preferably at least about 25%; yet more preferably at least about 50%; and most preferably at least about 75%. Optimally a therapeutically effective amount is that amount which when administered along with a chemotherapeutic agent according to the methods of the present invention results in complete remission of the tumor.

The gastrin anti-sense RNA expressed by the DNA molecule comprising a gastrin anti-sense sequence operatively linked to a regulatory sequence such that the DNA molecule expresses a gastrin anti-sense RNA in the tumor tissue according to the methods of the present invention may be from about 10 to about 400 nucleotides in length. In another embodiment, the gastrin anti-sense RNA may be from about 12 to about 50 nucleotides in length. In yet another embodiment, the gastrin anti-sense RNA may be from about 15 to about 25 nucleotides in length.

In a particularly useful embodiment, the method of the present invention includes administering a DNA vector that includes the above-described DNA molecule to a patient suffering from a gastrin-stimulated tumor. Such vectors have the advantage that they may be replicated in cell culture prior to administration according to the methods of the present invention.

In another aspect, the present invention includes administering an RNA vector that expresses the above-described anti-sense RNA molecule, along with a chemotherapeutic agent to a patient in need thereof. Alternatively, the above-described anti-sense RNA molecule may be administered directly with a chemotherapeutic agent.

The methods of the present invention also include administration, with a chemotherapeutic agent, of compositions including an RNA molecule comprising or expressing a gastrin anti-sense sequence via routes of administration including but not limited to intratumoral, aerosol, percutaneous, endoscopic, topical, intralesional, parenteral, and subcutaneous routes of administration. The nucleic acid molecule comprising a gastrin anti-sense sequence operatively linked to regulatory sequence that expresses a gastrin anti-sense RNA, or the anti-sense nucleic acid may be administered systemically or locally at the site of the tumor, or preferably injected into the tumor tissue.

The DNA molecule comprising a gastrin anti-sense sequence operatively linked to a regulatory sequence administered according to the methods of the present invention is preferably administered to the patient in a pharmaceutical composition.

The pharmaceutical compositions useful in the practice of the methods of the present invention include a DNA molecule comprising a gastrin anti-sense sequence operatively linked to a regulatory sequence that expresses a gastrin anti-sense RNA and a physiologically compatible carrier or excipient. Suitable carriers or excipients include any physiologically compatible buffer, such as, for instance phosphate buffered saline (PBS).

In another embodiment, the pharmaceutical compositions useful in the practice of the methods of the present invention include a nucleic acid molecule comprising a gastrin anti-sense sequence and a physiologically compatible carrier or excipient.

Chemotherapeutic agents useful in the practice of the present invention include: chemotherapeutic agents such as gemcitabine, camptothecin, doxorubicin, 5-fluorouracil (5FU), docetaxel, paclitaxel, vinblastine, etoposide (VP-16), oxaloplatin, carboplatin, cisplatin (CDDP), a kinase inhibitor (e.g. erlotinib, Iressa®), an angiogenesis inhibitor (e.g. Bevacizumab), and an EGF receptor inhibitor (e.g. cetuximab).

The chemotherapeutic agent administered with the DNA molecule comprising a gastrin anti-sense sequence operatively linked to a regulatory sequence according to the practice of the present invention may be administered at any stage of the anti-sense therapy regimen according to the methods of the present invention.

Thus, the chemotherapeutic agent may be administered continuously during treatment with the DNA molecule expressing a gastrin anti-sense RNA. Alternatively, the chemotherapeutic agent may be administered at intervals during treatment with the DNA molecule expressing a gastrin anti-sense RNA. In another regimen contemplated by the present invention, the chemotherapeutic agent may be

administered after treatment with the DNA molecule expressing a gastrin anti-sense RNA.

Similarly, the methods treatment of gastrin-promoted tumors of the present invention wherein an anti-sense nucleic acid (RNA or DNA) molecule is administered directly at the site of the tumor in conjunction with a chemotherapy regimen, include regimens wherein the chemotherapeutic agent may be administered at any stage of the anti-sense therapy regimen.

## **Materials and Methods**

### **Cell lines**

PAN1 is a human pancreatic cell line derived from a poorly differentiated human pancreatic adenocarcinoma (Academic Unit of Cancer Studies, University of Nottingham, UK). This cell line is a distinct form of the PANC1 cell line (ECACC No: 87092802).

BXPC3, a moderate to poorly differentiated human pancreatic cell line, and HCT116, a poorly differentiated human colon cell line, were obtained from the European collection of Cell Cultures (ECACC, Health Protection Agency, Porton Down, Salisbury, Wiltshire, SP4 0JG) and assigned reference nos. 93120816 and 91091005, respectively.

ST16 is a poorly differentiated human gastric adenocarcinoma cell line, derived within the Academic Unit of Cancer Studies (University of Nottingham, UK).

AR42J is a rat pancreatic adenocarcinoma cell line and was obtained from ECACC (Ref. No: 93100618).

All cell lines were routinely cultured in RPMI 1640 culture medium (Gibco, Paisley, UK) containing 10% (v/v) heat inactivated fetal bovine serum (FBS, Sigma, Poole, UK) at 37°C in 5% CO<sub>2</sub> and humidified conditions. For experimental use, cells from semi-confluent monolayers were harvested with 0.025% ethylenediaminetetraacetic acid (EDTA, Sigma, Poole, UK).

### **Human tumor specimens**

Fasting sera were obtained from pancreatic cancer patients, pre-operatively and stored at -80°C. Tumor tissue was obtained following resection and was either snap frozen in liquid nitrogen within 30mins and stored at -80°C or fixed in formalin and embedded into wax, prior to processing.

**Ethics:** All patients consented to the study and ethical approval was obtained from the Ethics Committee at the Queen's Medical Centre, University Hospital, Nottingham, UK.

### **Measurement of gastrin gene expression**

#### **Extraction of RNA**

Total RNA was extracted from frozen and fixed tissue, as previously described (McWilliams DF, et al. Coexpression of gastrin and gastrin receptors (CCK-B and  $\Delta$ CCK-B) in gastrointestinal tumor cell lines. *Gut* 42(6): 795-798, 1998).

#### **Real Time PCR**

RNA was reverse transcribed from random hexamer primers (Pharmacia) using Superscript RT (GibcoBRL, UK). Real Time PCR was performed using the 5700 Sequence Detection System (PE Applied Biosystems, Warrington, UK) as previously described (Watson SA & Smith AM. Hypergastrinaemia promotes adenoma progression in the APC<sup>Min/+</sup> mouse model of familial adenomatous polyposis. *Cancer Res.* 61: 625-631, 2001). The gastrin primer sequences were as follows:-

Upper strand primer CCACACCTCGTGGCAGAC (SEQ ID NO: 1).

Lower strand primer TCCATCCATCCATAGGCTTC (SEQ ID NO: 2).

The relative gene expression for each sample was determined using the formula

$$2^{-\Delta Ct} = 2^{-Ct(GAPDH) - Ct(gastrin)},$$

and reflected gastrin gene expression normalized to GAPDH levels.

#### **Immunohistochemical evaluation of progastrin expression**

Cells were cultured in 8-well SuperCell® chamber slides (Menzel-Gläser, Braunschweig, Germany) for 24hrs with RPMI (Sigma, Poole, UK) plus 10% fetal bovine serum (Sigma, Poole, UK) at a density of  $5 \times 10^4 \text{ ml}^{-1}$  then fixed in cold 70% ethanol at sub-confluence and stained with polyclonal rabbit anti progastrin antibodies raised against the amino(N)-terminal domain of progastrin (Aphtron Corporation, CA, USA) (Smith AM & Watson SA. Gastrin And Gastrin Receptor Activation - An Early Event In The Adenoma-Carcinoma Sequence. *Gut* 47: 820-824, 2000).

Progastrin labeling was assessed by computerized image analysis using custom macro-routines created with Qwin Standard analysis software (Lieca Microsystems, Cambridge UK). Results are represented as mean % labelling over an average of 15 readings per coverslip or tissue section. Inter-observer variation 6% and intra-assay variation <10%.

#### **Radio-immunoassay for progastrin and amidated gastrin**

Supernatants were collected from the cell lines as previously described and together with fasting patient sera were analyzed by radio-immunoassay for the presence of amidated gastrin-17, gastrin-34 and progastrin within the laboratories of Professor Andrea Varro, as previously described (Watson SA, et al. Gastrimmune

raises antibodies that neutralise amidated and glycine extended gastrin-17 and inhibit the growth of colon cancer. *Cancer Res* 56: 880-885, 1996).

***In vitro* clonogenicity assays**

Sub-confluent cells were harvested, re-suspended in RPMI+10%FBS at concentrations between  $1 \times 10^3$  and  $6 \times 10^3$  viable cells/ml and plated into 96-well plates in a final volume of 200 $\mu$ l in replicates of five. The total volume within each well was made up to 200 $\mu$ l with growth medium and the plates incubated at 37°C, 5% CO<sub>2</sub> for 18 hours. The medium was aspirated and replaced with 200 $\mu$ l of RPMI + 1%FBS. Cell numbers were assessed at 0, 24, 48 and 72 hours using a methyl thiazolyl tetrazolium (MTT) based assay as previously described (Watson SA et al. *Cancer Res* 56: 880-885, 1996).

***In vitro* growth assays with anti-gastrin antibodies and cytotoxics**

The cytotoxics, gemcitabine (Eli Lilly, UK), cisplatin (Rhone-Poulenc Rorer, UK), camptothecin (Faulding Pharmaceuticals, UK) and taxotere (Rhone-Poulenc Rorer) were prepared as stock solutions (prepared at 1mg/ml in sterile distilled water) and then diluted into assay medium (RPMI with 1% FBS).

Affinity purified mouse monoclonal anti-gastrin antibodies were raised against the amino-terminal of human gastrin-17 (anti-gastrin mAb, G17, Aphton Corporation, CA) and diluted to a final concentration of 100 and 500 $\mu$ g/ml in assay medium from a stock solution (2mg/ml in sterile PBS). Purified mouse IgG1 Kappa antibodies (Sigma) were used as a negative control. Sub-confluent cells were harvested, re-suspended in RPMI +10% FBS at a concentration of  $1 \times 10^5$  viable cells/ml and plated into 96-well plates in 100 $\mu$ l aliquots. The cells were incubated overnight at 37°C. The medium was aspirated and replaced by test compounds/antibodies with the final volume per well being 100 $\mu$ l. The plates were then incubated for 48 hours at 37°C and proliferation assessed by the MTT assay.

Additive and synergistic effects were defined as follows:-

IF ADDITIVE	$\frac{\text{Growth in presence of anti-gastrin mAb}}{\text{Growth in presence mouse IgG}}$	=	$\frac{\text{Growth in presence of anti-gastrin mAb + cytotoxic}}{\text{Growth in presence mouse IgG + cytotoxic}}$
IF SYNERGY	$\frac{\text{Growth in presence of anti-gastrin mAb}}{\text{Growth in presence mouse IgG}}$	<	$\frac{\text{Growth in presence of anti-gastrin mAb + cytotoxic}}{\text{Growth in presence mouse IgG + cytotoxic}}$

#### Gastrin antisense plasmid construction

PCR amplification of the entire coding region of the gastrin gene was carried out on cDNA from human LoVo colorectal cells using the gastrin sense primer CAGAGACCTGAGAGGCACCAG (SEQ ID NO: 3), and the gastrin antisense GTTCTAGGATGGTTAGTTCTCATC (SEQ ID NO: 4). The antisense primer was phosphorylated at the 5' terminus to facilitate cloning in the antisense direction.

The human gastrin cDNA sequence (NCBI accession number NM\_000805) is shown below as SEQ ID NO:5:

```

1 agcagagacc tgagaggcac caggcccagc cgtggcacca cacacctccc agctctgacg
61 acgagatgca gcgactatgt gtgtatgtgc tgatcttgc actggctctg gccgccttct
121 ctgaagcttc ttggaagccc cgctcccagc agccagatgc acccttaggt acagggggcca
181 acagggacct ggagctaccc tggctggagc agcagggccc agcctctcat catogaaggc
241 agctgggacc ccaggggtccc ccacacctcg tggcagaccc gtccaagaag cagggaccat
301 ggctggagga agaagaagaa gcctatggat ggatggactt cggccgccgc agtgctgagg
361 atgagaacta acaatcctag aaccaagctt cagagcctag ccacctccca cccacttca
421 gccctgtccc ctgaaaaact gatcaaaaat aaactagttt ccagtggatc

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The locations of binding site of the sense primer (nucleotides 3-23) and anti-sense primer (nucleotides 360-383) are underlined in the human gastrin cDNA sequence above. The product was cloned into the TA cloning vector pCR3.1 Uni (Invitrogen, Netherlands). Bacterial transformants were assayed by PCR and sequenced using forward and reverse primers to ensure that the insert was in the antisense orientation.

#### Tissue Culture and Transfection



Cells were seeded into 24-well plates, cultured until approximately 50% confluent and transfected in serum-free media using TFX-50 liposomes (Promega, UK) according the manufacturer's instructions. The transfection mix of 200µl containing 1µg plasmid and 4.5 µl liposomes was gently layered on the cells and incubated at 37°C for 60 minutes. Culture medium was added and the cells were placed in the incubator for 48 hours prior to selection with 1mg/ml G418 (Sigma, UK). After 4 weeks of selection the cells formed a monolayer and were confirmed as stable transfectants by PCR. The establishment of stable antisense and control plasmid (self-ligated) clones was achieved by plating cells at a density of less than one cell per well in a microtitre plate. Clonal colonies were established in selective media and expanded to form stable cultures.

#### **Caspase-3 assay of apoptosis**

Confluent cells were harvested, washed and counted. The cells were re-suspended into growth media (RPMI + 10%FBS) at  $1 \times 10^6$  viable cells/ml. 6 flasks were re-seeded per cell line at  $3 \times 10^6$  cells/flask. Total volume was made up to 15ml with growth media. The flasks were incubated overnight at 37°C, 5% CO<sub>2</sub>. Taxotere and gemcitabine solutions were prepared at 20 and 250µg/ml respectively, in assay medium (RPMI + 1%FBS + 2mM L-glutamine) and added individually to each cell line in duplicate. After 4 hrs incubation, the cells were harvested and the cell pellets assayed for caspase-3 activity using a Clontech assay kit (Clontech, Cowley, UK).

#### **Protein kinase B/Akt western blot analysis**

Following overnight serum starvation, cells were treated with fresh serum-free medium containing 10nM gastrin or 1% FBS medium containing gemcitabine or taxotere at their respective IC<sub>50</sub> concentrations for 1 hour. Following incubation, cells were washed with ice-cold PBS (Oxoid, Basingstoke, UK) and lysed with sample buffer [62.5mM Tris-HCl at pH 6.8 (Invitrogen), 2% w/v sodium dodecyl sulphate (SDS) (Sigma), 10% glycerol (Sigma), 50nM mercaptoethanol (Sigma), 0.01% w/v bromophenol blue (Sigma), 1mM sodium fluoride (Sigma), 80mM sodium B-glycerophosphate (Sigma), 1mM PMSF (Sigma) and 10µl/ml Calbiochem Protease Inhibitor Cocktail III (Calbiochem, San Diego, California, USA)].

Following primary antibody manufacturers instructions, samples were separated via gel electrophoresis on 8-16% Tris-glycine gels (Invitrogen), transferred to PVDF membrane (Invitrogen). Membranes were blocked and incubated with the primary polyclonal antibodies either Akt or phospho-Akt (Serine473) (New England Biolabs, Hitchin, UK), washed and then incubated with a HRP labelled swine anti-rabbit secondary antibody (Dako, Ely, UK) diluted to 1:1000. Chemiluminescent visualization and protein detection was carried out using

the Amersham ECL kit (Amersham, Little Chalfont, Buckinghamshire, UK) and Kodak X-OMAT film (Sigma).

***In vivo* therapeutic assays with antisense cell lines**

All tumors were grown in male MF1 nude mice (4-6 weeks of age) bred within the Academic Unit of Cancer Studies, University of Nottingham, UK. For orthotopic implantation, cells were re-suspended at  $1 \times 10^6$  in 20  $\mu$ l in sterile phosphate buffered saline, pH 7.4 (PBS). A laparotomy was performed under anaesthetic (Hypnorm, Roche: Hypnovel, Jannson) and the tip of the pancreas gently exteriorised before the cell inoculum was injected. The peritoneal wall was closed with suture and the skin with wound clips. Gemcitabine (Eli-Lilly Co. Ltd, Basingstoke, England) was administered at a dose of 16mg/kg, iv, on days 1,3,6 which was repeated at day 28 (as recommended by the manufacturer) and taxotere at a dose of 20mg/kg, iv, on day 15 (single dose) (Nicoletti M, et al. Comparison of paclitaxel and docetaxel activity on human ovarian carcinoma xenografts. *Eur J Cancer* 30A(5): 691-6, 1994). Experimental groups were between 10-15 mice.

All *in vivo* experimentation was performed according to the UK Coordinating Committee for Cancer Research (UKCCCR) guidelines.

**Statistics**

For evaluation of *in vitro* data, either a Students' t-test or a one way analysis of variance was used. Gene expression was assessed using a Mann Whitney non-parametric assessment and *in vivo* data was analysed by one-way analysis of variance using the Minitab statistical package.

**EXAMPLES**

**Example 1: Determination of gastrin expression in test cell lines, and comparison with human patient pancreatic adenocarcinomas.**

Gastrin gene expression was measured in two test pancreatic cell lines, PAN1 and BXPC3 by real time PCR and levels were in the range of those shown for a series of resected human pancreatic adenocarcinoma specimens (Figure 1a).

Expression at the level of protein was confirmed by determining progastrin immunoreactivity on the same series of human pancreatic adenocarcinoma specimens and the two test cell lines grown as xenografts. Progastrin expression by PAN1 and BXPC3 was shown to be at the upper and lower ranges, respectively, of the human pancreatic adenocarcinoma specimens (Figure 1b).

**Example 2: Progastrin and amidated gastrin secretion by human pancreatic tumor specimens.**

Tumor material from patients with locally advanced and metastatic pancreatic adenocarcinoma was not available due to the low rate of surgical resection in this patient group. In an attempt to indirectly demonstrate gastrin gene expression,

levels of both progastrin and amidated gastrin were determined in the serum of patients with either advanced or resectable disease.

In sera from 68 pancreatic adenocarcinoma patients, significantly greater levels of amidated gastrin and progastrin were detected in patients with advanced disease compared to patients with resectable disease ( $p=0.008$  and  $0.046$ , Student's t-test, Table 1).

**Table 1: Serum levels of progastrin and amidated gastrin in patients with resectable and advanced pancreatic adenocarcinoma**

	No. of patients (%) with detectable progastrin <sup>+</sup> (range pM)	No. of patients (%) with detectable amidated <sup>++</sup> gastrin
<b>Resectable pancreatic cancer</b>	0/17 (0%)	5/17 (29%)
<b>Advanced pancreatic cancer</b>	11/68 (16%)*	54/68 (79%)**

n= serum samples from 17 resectable and 16 advanced pancreatic patients

\*  $p=0.048$ , \*\*  $p=0.008$  when compared to resectable cancer, Student's t-test

Detection sensitivity of assay was + 40pM and ++ 4pM

**Example 3: Effect of neutralising gastrin antibodies on the basal growth of pancreatic cell lines alone and in combination with gemcitabine and taxotere**

The effect of 100 and 500 $\mu$ g/ml anti-gastrin mAbs on the basal growth of PAN1 and BXPC3 was determined by MTT uptake. An anti-gastrin mAb concentration of 100 $\mu$ g/ml significantly reduced basal growth of BXPC3 ( $p<0.0001$ , ANOVA, 10% inhibition when compared to IgG control) but not PAN1. An antibody concentration of 500 $\mu$ g/ml significantly inhibited the basal growth of both cell lines (12.7% inhibition with PAN1 and 22.9% with BXPC3,  $p<0.005$  when compared to IgG control, Table 2).

**Table 2: Synergistic effects of anti-gastrin monoclonal antibodies combined with the cytotoxic agents, taxotere and gemcitabine**

Cell line	% Inhibition {(anti-gastrin mAb + cytotoxics/mouse IgG + cytotoxics) x 100}		% Inhibition {(anti-gastrin mAb /mouse IgG) x 100}
	Taxotere	Gemcitabine	
PAN1	70.2 *	28.6**	12.7 <sup>+</sup>
BXPC3	50.0***	36.6	22.9 <sup>+</sup>

n=combined means of 2-3 separate assays, 5 replicates per assay

\*p<0.05, \*\*p<0.01, \*\*\*p<0.005, one-way analysis of variance when compared to % Inhibition of anti-gastrin mAb/mouse IgG

<sup>+</sup> p<0.005 when compared to mouse IgG alone

The higher concentration of anti-gastrin mAbs was combined with the cytotoxics, gemcitabine and taxotere, at their respective IC<sub>50</sub>s to determine whether synergistic effects on growth could be achieved (Table 2).

With BXPC3 cells, a taxotere concentration of 0.075µg/ml and gemcitabine concentration of 0.05µg/ml were defined as the respective IC<sub>50</sub> doses. Anti-gastrin mAbs significantly synergised with taxotere resulting in the inhibitory effect of the antibodies increasing from 22.9% to 50% following correction for the effect of the cytotoxic (p<0.005, ANOVA, Table 2). No significant synergy was observed with anti-gastrin mAbs in combination with gemcitabine.

With PAN1 cells, the IC<sub>50</sub> with taxotere was achieved at a concentration of 15.0µg/ml. The inhibitory effect of anti-gastrin mAbs increased from 12.7% to 70.2% following correction for the effect of cytotoxics alone (p<0.05, Table 2). PAN1 was highly resistant to the anti-proliferative effects of gemcitabine in cell culture and an IC<sub>50</sub> was not achieved. Synergy was examined using a concentration of 250µg/ml which induced a maximum inhibition of 18.0% and significant synergy was achieved with anti-gastrin mAbs with inhibition increasing from 12.0 to 28.6% (p<0.01, Table 2).

#### **Example 4: Characterisation of PAN1 cells transfected with an antisense gastrin gene construct**

Figure 2a shows the gastrin gene expression in PAN1 vector control (PAN1 VC) transfected cells in comparison to a PAN1 clone transfected with the gastrin antisense plasmid (PAN1 AS). There was a significant log-fold reduction of gastrin gene expression in the antisense cell line compared to the vector control (p<0.01,

Mann Whitney). Gastrin secretion was measured by assessing progastrin levels in supernatant concentrated from cells. Supernatant from the vector control cells secreted 58.0 pmols progastrin per  $8 \times 10^6$  cells compared to non-detectable levels in the gastrin antisense cell line.

An antibody raised against the N-terminus of progastrin was used to stain the cells to indicate gastrin immuno-reactivity and these results are shown in Figure 2b. Mean % staining in PAN1 VC was 52.12 and 49.66 (2 separate assays) compared to 4.91 and 2.19 in PAN1 AS ( $p < 0.0001$ , Student's t-test).

Clonogenic assays were performed with PAN1 VC and AS in both 1% and 0.2% serum-containing growth medium (Figure 2c). In 1% serum, PAN1 VC had significantly greater growth at the 144 and 168 hour time points ( $p < 0.01$ , Student's t-test). In the 0.2% serum concentration, PAN1 VC cells grew modestly whereas PAN1 AS cells failed to grow with significantly lower MTT absorbance compared to PAN1 VC at all time points after 72 hours ( $p < 0.01$ ).

**Example 5: Sensitivity of PAN1 vector control and antisense cell lines to cytotoxic agents *in vitro***

The effect of abrogation of the gastrin gene on the sensitivity of PAN1 VC and AS cells to taxotere and gemcitabine was assessed by *in vitro* proliferation and typical dose-response curves (from a series of 2-3 repeats per cell line) are shown in Figures 3a and b.

The PAN1 AS cell line was more sensitive to the anti-proliferative effects of gemcitabine with the  $IC_{50}$  achieved at approximately  $0.1 \mu\text{g/ml}$  compared to  $>100 \mu\text{g/ml}$  with PAN1 VC ( $IC_{50}$  at approximately  $250 \mu\text{g/ml}$  but was difficult to achieve due to flat dose response curve at gemcitabine doses  $>100 \mu\text{g/ml}$ ). At gemcitabine concentrations between  $0.1$ - $100 \mu\text{g/ml}$  there was significantly more inhibition ( $p < 0.000$ , ANOVA) observed with PAN1 AS when compared to PAN1 VC cells (Figure 3a).

The  $IC_{50}$  with taxotere was approximately  $20 \mu\text{g/ml}$  in PAN1 VC compared to  $<0.01 \mu\text{g/ml}$  in PAN1 AS. There was a significant difference in inhibitory effects between the two cell lines at all taxotere concentrations (Figure 3b).

**Example 6: Sensitivity of PAN1 vector control and antisense cell lines to cytotoxic agents *in vivo***

PAN1 VC and AS cells were grown as xenografts orthotopically in the pancreas of nude mice and treated with therapeutic doses of the cytotoxic agents, taxotere and gemcitabine. The final pancreatic tumor cross-sectional areas and weights are shown in Figures 4a and b, respectively.

Both taxotere and gemcitabine significantly inhibited the cross-sectional area of PAN1 VC xenografts by 31.6% ( $p=0.040$ , ANOVA) and 61.2% ( $p=0.000$ ), respectively (Figure 4a). The mean cross-sectional area of PAN1 AS xenografts was reduced by 94% ( $p=0.000$ ) when compared to PAN1 VC with tumors detectable in 8/15 mice. When PAN1 AS xenografts were treated with cytotoxics, 1/10 tumors grew in the taxotere treated group ( $p=0.013$  compared to control-treated PAN1 AS) and 0/10 in the gemcitabine treated group ( $p=0.009$ ).

When assessing final tumor weights (Figure 4b) similar trends were observed, gemcitabine inhibited the weights of PAN1 VC xenografts ( $p=0.000$ , ANOVA) whereas the effect of taxotere failed to reach statistical significance ( $p=0.115$ ). PAN1 AS xenografts were significantly smaller than PAN1 VC xenografts ( $p=0.000$ ) and taxotere and gemcitabine treated PAN1 AS tumors had significantly lower mean tumor weights when compared to PAN1 AS control treated xenografts ( $p=0.03$  and  $p=0.006$ , respectively).

**Example 7: Apoptotic activity of taxotere and gemcitabine in PAN1 VC and PAN1 AS transfected cell lines**

Apoptosis was detected by measurement of caspase-3, following short-term (4 hours) treatment with taxotere and gemcitabine. When comparing the level of apoptosis in untreated PAN1 VC versus PAN1 AS cells, the levels were not significantly different ( $p=0.37$ , ANOVA, Figure 5). However, the levels of caspase-3 following treatment with taxotere and gemcitabine were significantly higher in PAN1 AS cells when compared to PAN1 VC ( $p=0.032$  and  $0.013$ , respectively, Figure 5).

**Example 8: Protein kinase B/Akt expression/phosphorylation in PAN1 VC and PAN1 AS cell lines following treatment with taxotere and gemcitabine:** The basal PKB/Akt expression and phosphorylation status of the PAN1 vector control and gastrin antisense cell lines are shown in Figure 6a. The PAN1 vector control cell line showed higher basal levels of phosphorylated PKB/Akt than the control cell line (AR42J). The basal levels of phosphorylated PKB/Akt were reduced in the gastrin antisense cell line and both cell lines were shown to respond modestly to gastrin stimulation in serum free medium as determined by increased phosphorylation which peaked at 30 minutes in PAN1 VC and 60 minutes in PAN1 AS cell extracts (Figure 6a).

To determine the effects of cytotoxic treatment on PKB/Akt phosphorylation levels, cells were grown in 1% serum-containing medium and treated with either taxotere or gemcitabine for 1 hour. In the gastrin antisense cell line, phosphorylated PKB/Akt levels were reduced following taxotere treatment, unlike the PAN1 VC cells (Figure 6b).

Trypan blue viability assays were carried out on the PAN1 cells following 1 hour treatment with the cytotoxics to ensure changes in PKB/Akt phosphorylation were not due to a reduction in cell number. There was no viability loss in either cell line.

**Example 9: Sensitivity of a panel of gastrin antisense transfected GI cell lines to a series of cytotoxic agents:** Stable gastrin antisense transfectants of HCT116 and ST16 were derived and together with PAN1 *in vitro* sensitivity to a wider panel of cytotoxics was determined (Table 3). Significantly increased sensitivity to taxotere was observed in all 3 gastrin antisense cell lines when compared to their corresponding VC lines ( $p \geq 0.007$ , ANOVA, Table 3). Increased sensitivity was also seen with camptothecin for all gastrin AS-transfected lines. However, only PAN1 AS retained increased sensitivity to cisplatin with similar sensitivity shown between the vector control and gastrin antisense in the gastric and colorectal cell lines (Table 3).

**Table 3: Cytotoxic concentrations inducing 50% growth inhibition in a vector control and gastrin antisense cell line panel**

Cell line		IC <sub>50</sub> (µg/ml) for each cytotoxic		
		Taxotere	Cisplatin	Camptothecin
PAN1	VC	20.0	3.0	60.0
	AS	0.01 <sup>1</sup>	0.30 <sup>1</sup>	3.0 <sup>2</sup>
HCT116	VC	0.020	2.0	30.0
	AS	0.003 <sup>5</sup>	2.5 <sup>3</sup>	9.0 <sup>4</sup>
ST16	VC	10.0 <sup>1</sup>	4.0	80.0
	AS	0.006 <sup>1</sup>	3.0 <sup>3</sup>	20.0 <sup>1</sup>

Results for a single representative experiment, 5 replicates per assay

Statistical significance from VC cell line determined by ANOVA at concentration relating to IC<sub>50</sub>, <sup>1</sup> $p=0.000$ , <sup>2</sup> $p=0.003$ , <sup>3</sup>NS, <sup>4</sup> $p=0.006$ , <sup>5</sup> $p=0.007$

The above examples demonstrate that reduction of gastrin gene expression in PAN1 cells by gastrin antisense RNA (PAN1 AS) significantly reduced growth in 1% serum by approximately 40% and completely suppressed growth in 0.2% serum-containing medium. *In vivo* PAN1 AS cells were poorly tumorigenic when transplanted orthotopically with a 93% reduction in final tumor size and weight and almost complete elimination of tumor growth following treatment with either taxotere

or gemcitabine. The present study showed that sensitivity to cytotoxic agents was increased when comparing  $IC_{50}$  values achieved with taxotere, gemcitabine, camptothecin and cisplatin were significantly lowered in PAN1 AS compared to PAN1 VC; the greatest sensitivity being to taxotere. This effect was not limited to the PAN1 cell line. A colorectal cell line (HCT116) and a gastric cell line (ST16) also exhibited increased sensitivity to taxotere and camptothecin, (but not cisplatin) following stable transfection with gastrin antisense plasmid.



We claim:

1. A method of treating a patient suffering from a gastrin-promoted tumor, comprising administering to the patient (a) a therapeutically effective amount of a nucleic acid molecule comprising a gastrin anti-sense sequence operatively linked to a regulatory sequence that expresses a gastrin anti-sense RNA, and (b) a chemotherapeutic agent.
2. The method according to claim 1, wherein the gastrin-promoted tumor is an advanced stage tumor, or a gastrin-secreting tumor, or both.
3. The method according to claim 1, wherein the chemotherapeutic agent comprises one or more of the following: gemcitabine, camptothecin, doxorubicin, 5-fluorouracil (5FU), docetaxel, paclitaxel, vinblastine, etoposide (VP-16), oxaloplatin, carboplatin, cisplatin (CDDP), a kinase inhibitor, an angiogenesis inhibitor, and an EGF receptor inhibitor.
4. The method according to claim 1, wherein the gastrin-promoted tumor is a tumor of the gastrointestinal (GI) tract.
5. The method according to claim 4, wherein the gastrin-promoted tumor of the gastrointestinal (GI) tract is a tumor of the esophagus, a tumor of the gastric tissue, a tumor of the intestine or a tumor of the colorectal tract.
6. The method according to claim 5, wherein the gastrin-promoted tumor of the colorectal tract is a colonic adenoma.
7. The method according to claim 1, wherein the gastrin-promoted tumor is a pancreatic tumor, a medullary thyroid carcinoma (MTC), a glioblastoma, an ovarian tumor, or a tumor of neuroendocrine origin.
8. The method according to claim 1, wherein the gastrin-promoted tumor is a tumor of the lung.
9. The method according to claim 8, wherein the gastrin-promoted tumor of the lung is a small cell lung cancer (SCLC).
10. The method according to claim 1, wherein the gastrin-promoted tumor is a metaplasia or a dysplasia.
11. A method of treating a patient suffering from a gastrin-promoted tumor, comprising administering to the patient (a) a therapeutically effective amount of a single stranded nucleic acid molecule comprising a gastrin anti-sense sequence, and (b) a chemotherapeutic agent.
12. A gastrin anti-sense expression vector, comprising the human gastrin gene sequence from about nucleotide 3 to about nucleotide 383 of SEQ ID NO:5, and/or the complement of the human gastrin gene sequence from about nucleotide 3 to about nucleotide 383 of SEQ ID NO:5, wherein the vector expresses the anti-sense

sequence complementary to the human gastrin gene sequence from about nucleotide 3 to about nucleotide 383 of SEQ ID NO:5.

13. A method of making a gastrin anti-sense expression vector, comprising incorporating the human gastrin gene sequence from about nucleotide 3 to about nucleotide 383 of SEQ ID NO:5, and/or the complement of the human gastrin gene sequence from about nucleotide 3 to about nucleotide 383 of SEQ ID NO:5 into an expression vector, wherein the vector expresses the anti-sense sequence complementary to the human gastrin gene sequence from about nucleotide 3 to about nucleotide 383 of SEQ ID NO:5.
14. A pharmaceutical composition comprising a nucleic acid molecule comprising a gastrin anti-sense sequence operatively linked to a regulatory sequence that expresses a gastrin anti-sense RNA, and a pharmaceutically acceptable carrier.
15. A pharmaceutical composition comprising a nucleic acid molecule comprising a gastrin anti-sense sequence, and a pharmaceutically acceptable carrier.
16. A method of treating a patient suffering from a tumor of the gastrointestinal (GI) tract, a pancreatic tumor, or a tumor of neuroendocrine origin, the method comprising administering to the patient (a) a therapeutically effective amount of a nucleic acid molecule comprising a gastrin anti-sense sequence operatively linked to a regulatory sequence that expresses a gastrin anti-sense RNA, and (b) a chemotherapeutic agent.
17. A method of treating a patient suffering from a tumor of the gastrointestinal (GI) tract, a pancreatic tumor, a medullary thyroid carcinoma (MTC), a tumor of the lung, a glioblastoma, an ovarian tumor, or a tumor of neuroendocrine origin, the method comprising administering to the patient (a) a therapeutically effective amount of a single stranded nucleic acid molecule comprising a gastrin anti-sense sequence, and (b) a chemotherapeutic agent.
18. A method of treating a patient suffering from a tumor comprising cells bearing a gastrin receptor, the method comprising administering to the patient (a) a therapeutically effective amount of a nucleic acid molecule comprising a gastrin anti-sense sequence operatively linked to a regulatory sequence that expresses a gastrin anti-sense RNA, and (b) a chemotherapeutic agent.
19. A method of treating a patient suffering from a tumor comprising cells bearing a gastrin receptor, comprising administering to the patient (a) a therapeutically effective amount of a single stranded nucleic acid molecule comprising a gastrin anti-sense sequence, and (b) a chemotherapeutic agent.

Figure 1 A

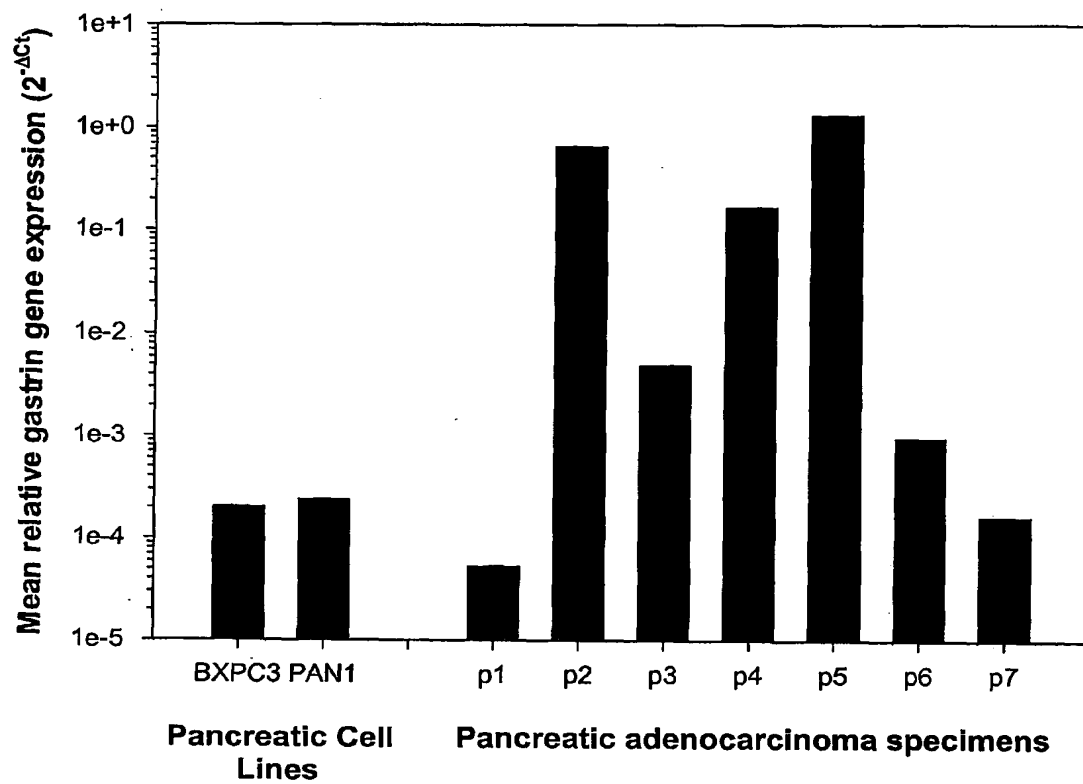


Figure 1 B

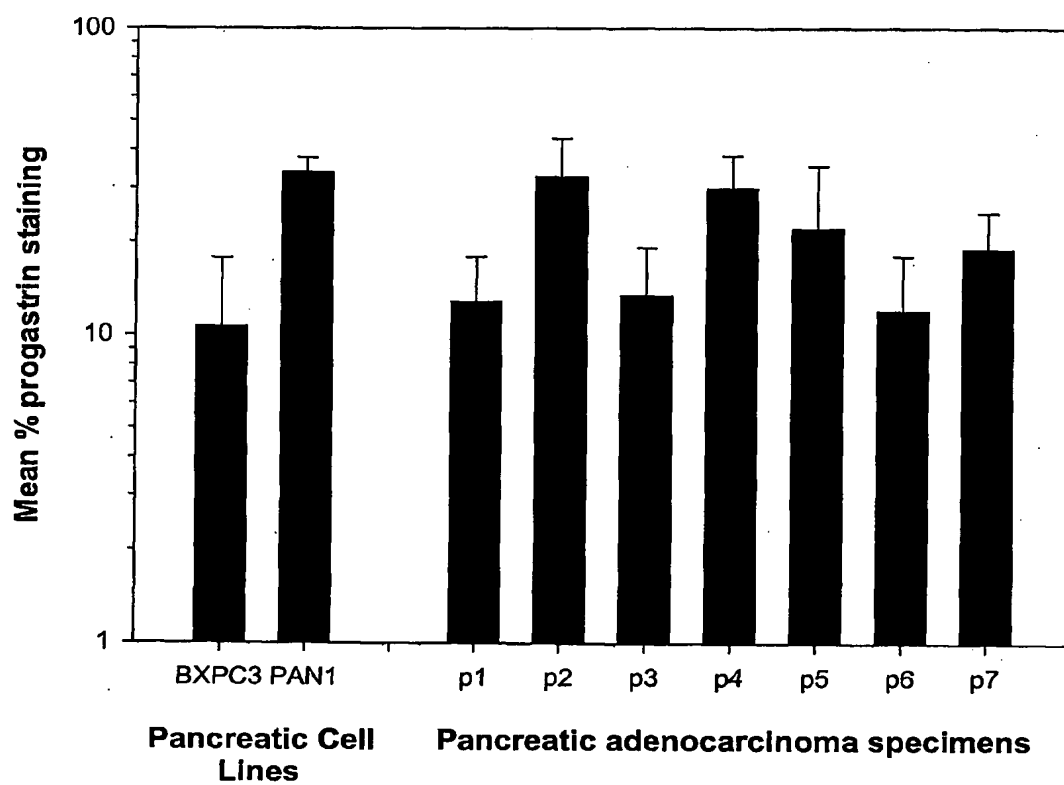


Figure 2

A

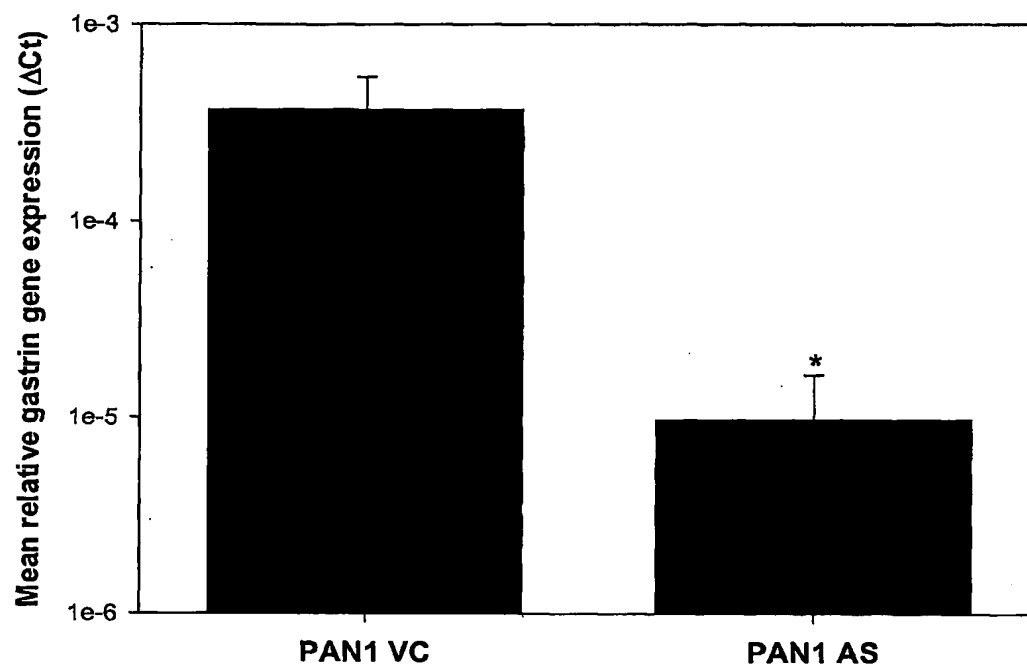


Figure 2B

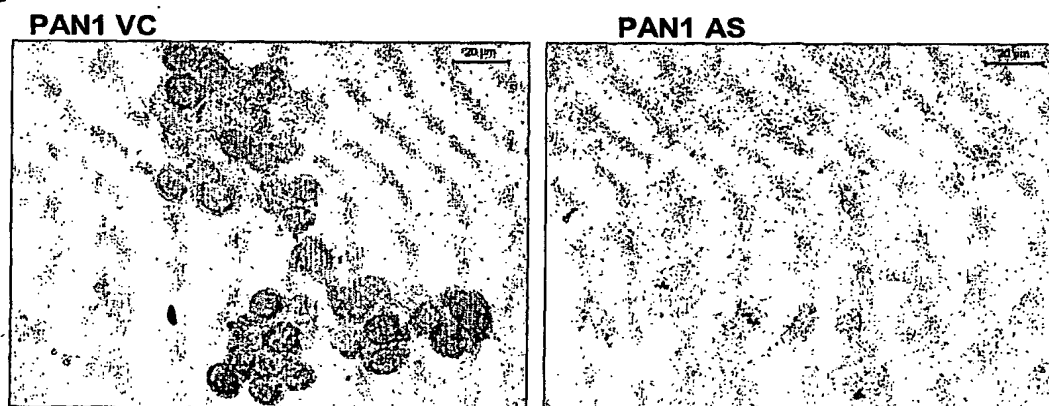
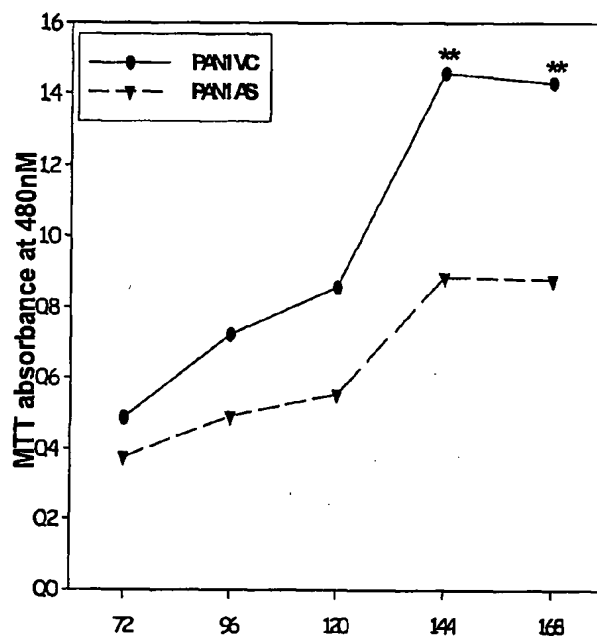


Figure 2C

## i) 1% serum containing medium



## ii) 0.2% serum containing medium

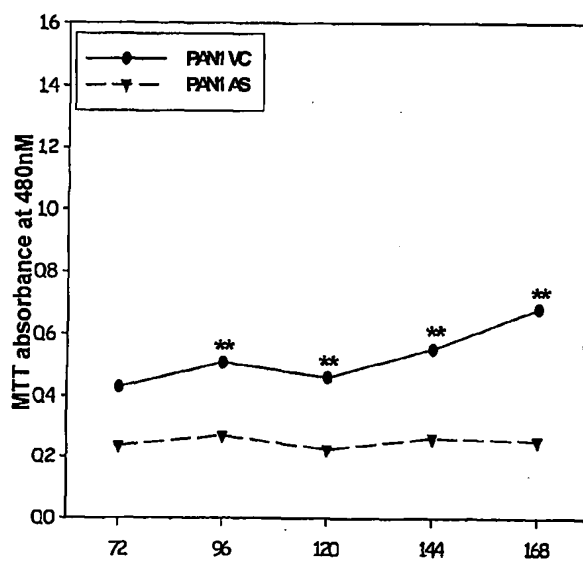


Figure 3 A

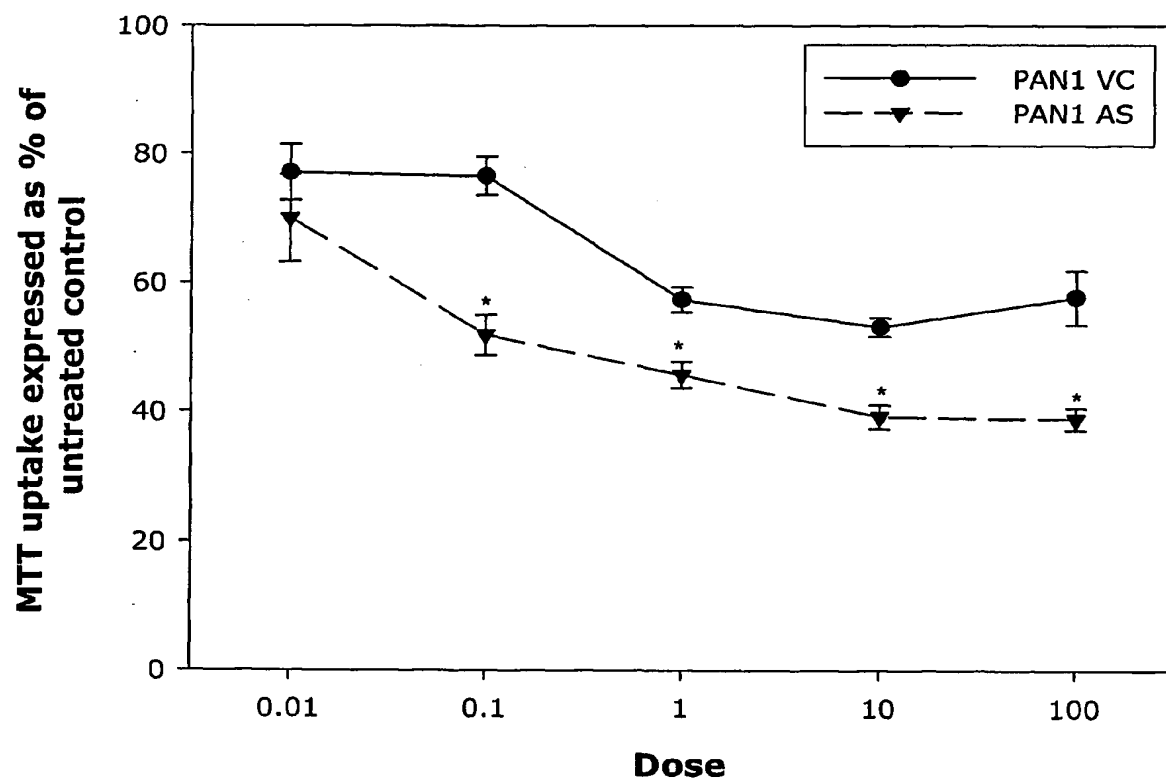
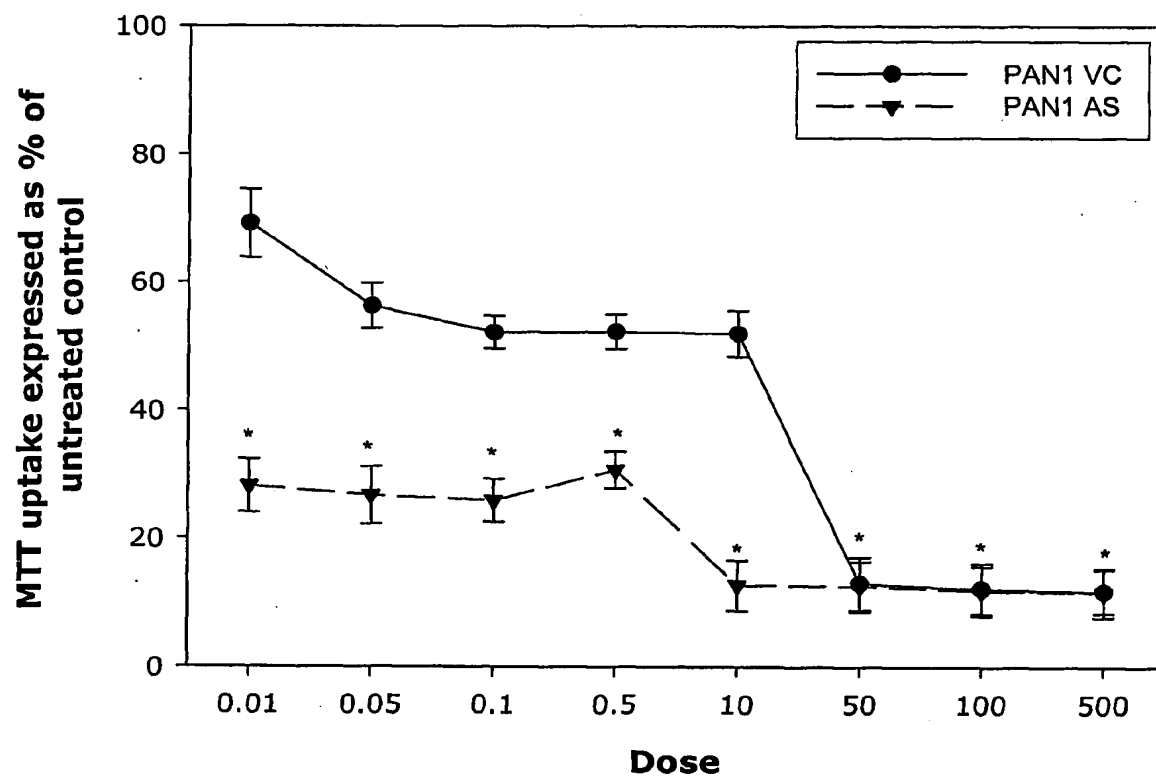


Figure 3B





**Figure 4**  
**A**

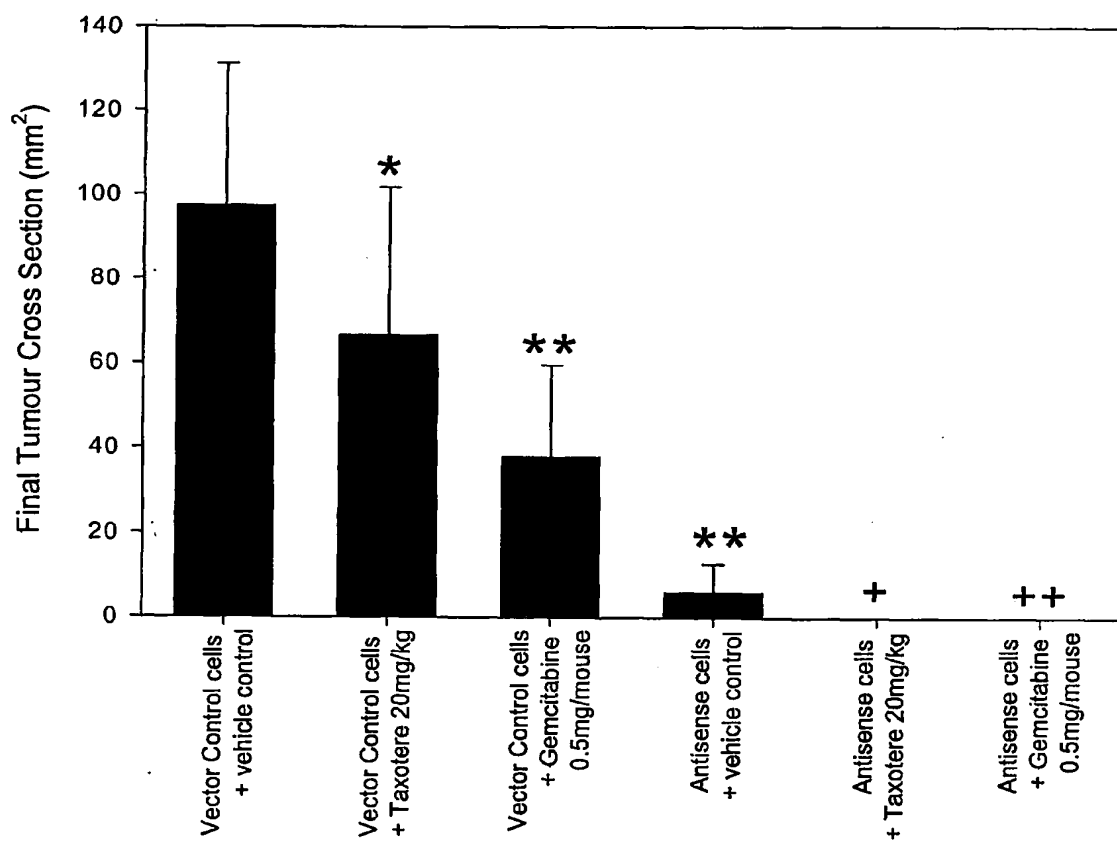


Figure 4 B

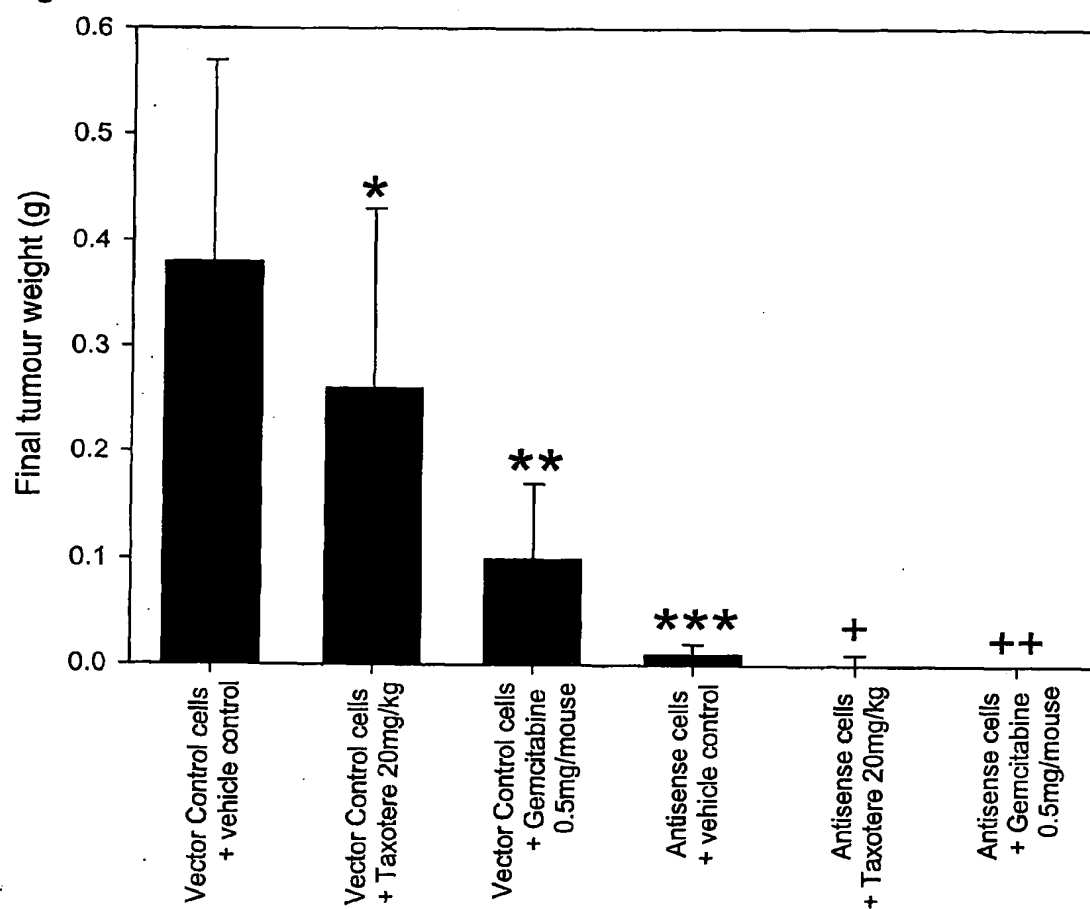


Figure 5

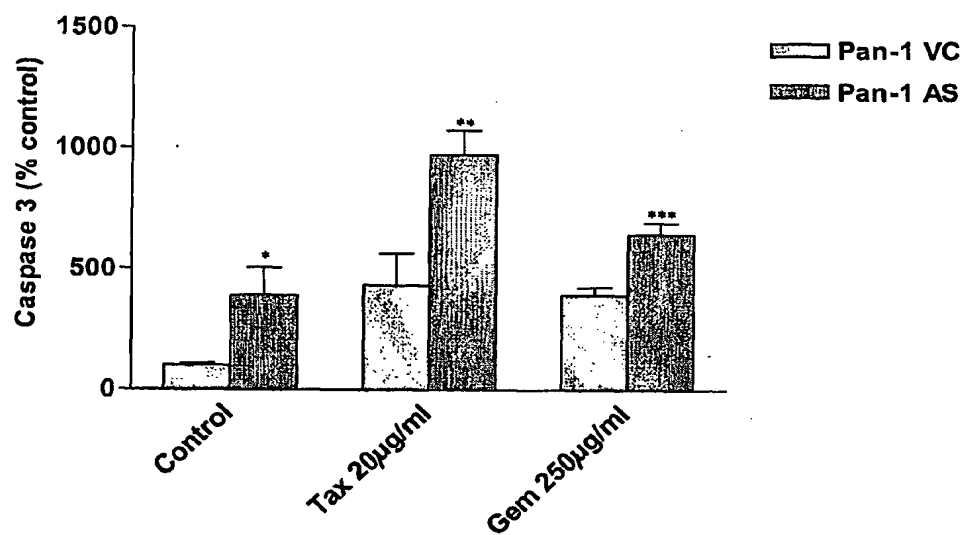
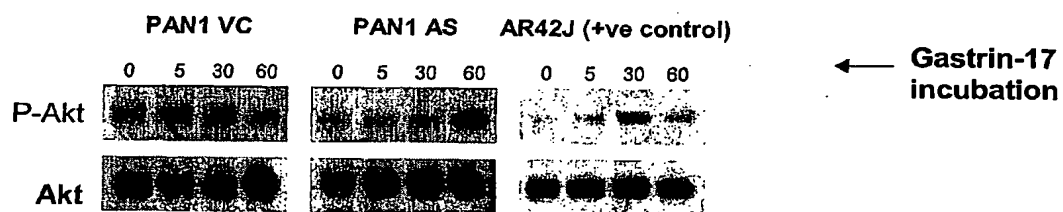
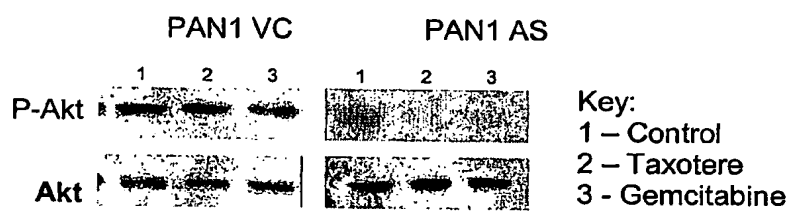


Figure 6

A



B



Aphton 0071.workFile.txt

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-----  
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 FaxNumber :  
 EmailAddress :  
 <110> OrganizationName : Aphton Corp.

## Application Project

-----  
 <120> Title : Anti-sense polynucleotide therapy for gastrin-promoted tumors  
 <130> AppFileReference : Aphton/0071  
 <140> CurrentAppNumber :  
 <141> CurrentFilingDate : \_\_\_\_-\_\_-\_\_

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 SequenceDescription :

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 SequenceDescription :

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 ctgaagcttc ttggaagccc cgtcccagc agccagatgc acccttaggt acaggggcca 180  
 acagggacct ggagctaccc tggctggagc agcagggccc agcctctcat catcgaaggc 240  
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Apton 0071.WorkFile.txt

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ggctggagga agaagaagaa gcctatggat ggatggactt cggccgccgc agtgctgagg 360
atgagaacta acaatcctag aaccaagctt cagagcctag ccacctcca cccacttca 420
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<211> Length : 470
      SequenceName : human gastrin cDNA sequence
      SequenceDescription :
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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/IB2005/002222

## A. CLASSIFICATION OF SUBJECT MATTER

C12N15/11 C07K14/595 C12N15/16 A61K48/00 A61K31/7088  
A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N C07K A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	WATSON S. ET AL.: "Gastrin inhibition increases the potency of cytotoxic agents in pancreatic cancer" GASTROENTEROLOGY, vol. 122, no. 4.supp1.1, April 2002 (2002-04), page A241, XP008056958	1-3,7, 12-18
Y	Abstract M952 the whole document	4-11,16, 17,19
X	WO 95/21380 A (LUDWIG INSTITUTE FOR CANCER RESEARCH (US); BALDWIN GRAHAM SHERARD) 10 August 1995 (1995-08-10) page 46; claims 38-40	12-15
Y	the whole document	4-10,16, 17
	----- -/--	

☒ Further documents are listed in the continuation of box C

☒ Patent family members are listed in annex

\* Special categories of cited documents .

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "&" document member of the same patent family

Date of the actual completion of the international search

8 December 2005

Date of mailing of the international search report

29/12/2005

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Authorized officer

Macchia, G

## INTERNATIONAL SEARCH REPORT

International Application No  
PCT/IB2005/002222

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No
X	WO 97/38584 A (BOARD OF REGENTS, THE UNIVERSITY OF TEXAS SYSTEM (US) SINGH P; WOOD TG) 23 October 1997 (1997-10-23)	12-15
Y	page 54; claim 12	4-10,16, 17
	the whole document	
Y	----- SMITH J.P. ET AL.: "Antisense oligonucleotides to gastrin inhibit growth of human pancreatic cancer" CANCER LETTERS, vol. 135, no. 1, 8 January 1999 (1999-01-08), pages 107-112, XP002357825 the whole document	11,19
Y	----- NÈGRE F. ET AL.: "Autocrine stimulation of ARA-2J rat pancreatic tumor cell growth by Glycine-extended Gastrin" INTERNATIONAL JOURNAL OF CANCER, vol. 66, no. 5, 1996, pages 653-658, XP008056928 the whole document	11,19
	-----	



# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/IB2005/002222

## Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons.

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
**Although claims 1-11, 16-19 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.**
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this International application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/IB2005/002222

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9521380	A	10-08-1995	NONE	
WO 9738584	A	23-10-1997	AU 2677097 A US 6165990 A US 5786213 A	07-11-1997 26-12-2000 28-07-1998